UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERISATION OF BACTERIOCIN PRODUCED BY LACTOCOCCUS LACTIS SUBSP.LACTIS RW18 ISOLATED FROM STEAMED FISH (RASTRELLIGER SP.)

LEE YOCK ANN

FSMB 2002 29

PURIFICATION AND CHARACTERISATION OF BACTERIOCIN PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS RW18* ISOLATED FROM STEAMED FISH (*RASTRELLIGER* SP.)

By

LEE YOCK ANN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

October 2002

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PURIFICATION AND CHARACTERISATION OF BACTERIOCIN PRODUCED BY *LACTOCOCCUS LACTIS* SUBSP. *LACTIS RW18* ISOLATED FROM STEAMED FISH (*RASTRELLIGER* SP.)

By

LEE YOCK ANN

October 2002

Chairman : Foo Hooi Ling, Ph.D.

Faculty : Food Science and Biotechnology

In this study, eight Lactic Acid Bacteria (LAB) isolated from "Ikan Rebus" (steamed fish) were screened for bacteriocin production using spot-on-lawn, flip streak plate and agar-well diffusion methods. Seven out of eight LAB isolates were confirmed to be able to produce bacteriocin. However, only the highest bacteriocin producer, RW 18, was selected for further studies. The carbohydrate fermentation pattern of RW 18 isolate exhibited 83.4% similarity to *Lactococcus lactis* subsp. *lactis* by the API CHL 50 test kit and hence designated as *Lc. lactis* subsp. *lactis RW18*. Bacteriocin production by *Lc. lactis* subsp. *lactis RW18* was detected during mid log phase and reached a maximum level of 200 Au/ml during the early stationary phase. Bacteriocin of *Lc. lactis* subsp. *lactis RW18* was able to tolerate wide pH range (pH 3.0 to pH 7.0) but it was unstable when the incubation temperature was increased above 90°C at pH 6.5. The bacteriocin demonstrated a

broad-spectrum antagonistic activity against gram-positive bacteria including *Listeria monocytogenes, Enterococcus faecalis, Enterococcus faecium, Pediococcus acidilactici* and *Lactobacillus pentosus* but it was not active against gram-negative bacteria. Results obtained in the study on the effect of hydrolytic enzymes indicated that the bacteriocin was a proteinaceous compound and most likely to contain lipolytic and glycolytic moieties. The bacteriocin was purified to homogeneity by a procedure involving 0-60% ammonium sulfate precipitation, cation-exchange chromatography and gel filtration chromatography with a yield of 0.9% and purification fold of 3210. The molecular mass of purified bacteriocin was estimated to be 3.9 kDa and 4.0 kDa using the Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE) and gel filtration chromatography respectively. The isoelectric point of the purified bacteriocin was estimated to be more than 9.30 by Isoelectric focusing-PAGE and hence it demonstrated a strong basic (cationic) characteristic. The stability of purified bacteriocin could be improved by adding either BSA or glycerol. A 100 % increment of relative activity was obtained by adding 10-40 µg of BSA, whereas a highest relative activity of 300 % was achieved when 10 % and 15 % of glycerol were added respectively. The purified bacteriocins have less antagonistic activity compared to crude bacteriocins. Partially purified bacteriocin pooled from the Resource-S chromatography exhibited enhanced biological activity against LAB, whereas reduced biological activity was observed for purified bacteriocin pooled after superose-12 gel filtration chromatography. NisA gene was detected in *Lc. lactis* subsp. *lactis RW18* by PCR amplification using a pair of *nisA* structural gene specific primers. The RAPD-PCR fingerprinting analysis revealed that *Lc. lactis*

subsp. *lactis RW18* was genotypically different from nisin producer, *Lc. lactis* subsp. *lactis ATCC 11454.* Nevertheless, evidence obtained in this study could not prove that the bacteriocin produced by *Lc. lactis* subsp. *lactis RW18* was nisin, regardless of the fact that *nisA* gene was detected in the *Lc. lactis* subsp. *lactis RW18*. The actual amino acid sequence of the purified bacteriocin has to be determined in order to ascertain whether bacteriocin produced by *Lc. lactis* subsp. *lactis RW18* is nisin.

Abstrak ini dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENULENAN DAN PENCIRIAN BAKTERIOSIN DIHASILKAN DARIPADA *LC. LACTIS* SUBSP. *LACTIS RW18* DIPENCILKAN DARI IKAN REBUS (*RASTRELLIGER* SP.)

Oleh

LEE YOCK ANN

Oktober 2002

Pengerusi : Foo Hooi Ling, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Dalam penyelidikan ini, lapan Bakteria Asid Laktik (BAL) yang dipencilkan daripada ikan rebus disaring untuk penghasilan bakteriosin dengan menggunakan kaedah "bintik atas gugusan", "plat terbalik berjalur" dan "difusi sumur agar". Tujuh daripada lapan pencilan BAL dipastikan berkemampuan menghasilkan bakteriosin. Walaubagaimanapun, hanya penghasil bakteriosin yang tertinggi sahaja, RW18 dipilih untuk kajian seterusnya. Corak fermentasi karbohidrat pencilan RW18 memaparkan 83.4% persamaan dengan *Lc. lactis* subsp. *lactis RW18* dengan kit ujian API 50 CHL. Maka it, pencilan RW18 dinamakan sebagai *Lc lactis* subsp. *lactis RW18*. Penghasilan bakteriosin dari *Lc lactis* subsp. *lactis RW18* mula dikesan semasa fasa pertengahan logaritma dan mencapai tahap maksimum sebanyak 200 AU/ml pada awal fasa pegun. Bakteriosin *Lc. lactis* subsp. *lactis RW18* berkeupayaan bertoleransi pada julat pH yang besar (pH 3.0 ke pH 7.0) tetapi ia

tidak stabil apabila suhu pengeraman meningkat lebih daripada 90 °C pada pH 6.5. Bakteriosin ini menunjukkan aktiviti spektrum antagonistik yang luas terhadap bakteria gram positif termasuk *Listeria monocytogenes, Enterococcus faecalis, Enterococcus facium, Pediococcus acidilactici* dan *Lactobacillus pentosus* tetapi ia tidak aktif terhadap bakteria gram negatif. Keputusan yang diperolehi dalam kajian kesan enzim hidrolitik menunjukkan bakteriosin ini adalah satu sebatian yang berprotein dan berkemungkinan mengandungi unsur-unsur lipolitik dan glikolitik. Bakteriosin ini ditulenkan sehingga mencapai tahap kesebakaan dengan satu prosedur yang melibatkan 0-60% pemendakan amonium sulfat, kromatografi penukar kation dan kromatografi penurasan gel. Kaedah penulenan ini telah menyebabkan 0.9% hasil dan 3210 tahap penulenan. Jisim molekul bagi bakteriosin yang ditulenkan dianggarkan sebanyak 3.9 kDa dan 4.0 kDa melalui kaedah analisa gel elektroforesis trisine poliakrilamid sodium dodesil sulfat dan kaedah kromatografi penurasan gel masing-masing. Titik isoelektrik bakteriosin yang ditulenkan adalah dianggarkan lebih daripada 9.30 dengan gel elektroforesis poliakrilamid pemusatan isoelektrik dan dengan ini menunjukkan ciri kationik yang kuat. Kestabilan bakteriosin yang ditulenkan boleh dibaiki dengan samada penambahan BSA atau gliserol. Sebanyak 100% penambahan aktiviti relatif boleh didapati dengan menambahan 10-40 μg BSA manakala aktiviti relatif setinggi 300% dicapai apabila 10% dan 15% gliserol ditambahkan masing-masing. Bakteriosin yang ditulenkan mempunyai activiti antagonistic yang rendah berbanding dengan bakteriosin kasar. Bakteriosin yang separa tulen yang dikumpul daripada kromotografi Resourse-S menunjukkan penambahan aktiviti biologi terhadap BAL yang diuji manakala pengurangan aktiviti biologi dikesan bagi bakteriosin tulen

yang dikumpulkan selepas kromatografi penurasan gel superose-12. Gen *nisA* dikesan dalam *Lc. lactis* subsp. *lactis RW18* dengan cara Amplifikasi Tindakbalas Berantai Polimerase oleh sepasang primer specifik gen struktur nisA. Analisa polimerifik DNA yang diamplikasikan secara rawak menunjukkan *Lc. lactis* subsp. *lactis RW18* adalah berbeza secara genetiknya daripada penghasil nisin, *Lc. lactis* subsp. *lactis ATCC 11454*. Namum demikian, bukti yang didapati daripada penyelidikan ini tidak dapat membuktikan bakteriosin yang dihasilkan dari *Lc. lactis* subsp. *lactis RW18* adalah nisin, walaupun gen nisA dikesan dalam *Lc. lactis* subsp *lactis RW18*. Jujukan asid amino yang sebenar bagi bakteriosin yang tulen perlu ditentukan untuk memastikan samada bakteriosin yang dihasilkan dari *Lc. lactis* subsp. *lactis RW18* adalah nisin.

ACKNOWLEDGEMENTS

First and foremost, I would like to take this opportunity to extend my deepest appreciation and gratitude to my project supervisor, Dr. Foo Hooi Ling for her invaluable help, advice, and guidance throughout my study.

I also would like to acknowledge the kindness of my co-supervisor, Prof. Dr. Gulam Rusul Rahmat Ali, providing Graduate Research Assistantship (GRA) for me to pursue Master degree. My gratefulness is also extending to my co-supervisor, Associate Prof. Dr. Son Radu for his advice and guidance.

Appreciation is extended to Prof. Dr. Hasanah Mohamed Ghazali and Dr. Raha Abdul Rahim of Biotechnology Department, UPM and Dr. Loh Teck Chwen of Animal Science Department, UPM for their courtesy allowing me to use their laboratory facilities.

I am grateful to all co-operative staffs in the Faculty of Food Science and Biotechnology, UPM especially En. Rosli and En. Halim.

I would like to convey my grateful to all my colleagues in Microbiology Laboratory for their encouragement and friendship especially Tik, Yin Sze, Kwan Kit, Chee Keong, Kak Zaleha, yousR, Wai Ling, Kqueen and Sam.

Lastly, I would like to dedicate this thesis to my beloved family who was wonderfully supportive of my studies.

I certify that an Examination Committee met on 26th October 2002 to conduct the final examination of Lee Yock Ann on her Master of Science thesis entitled "Purification and Characterisation of Bacteriocin Produced by *Lactococcus lactis* subsp. *Lactis RW18* Isolated from Steamed Fish (*Rastrelliger* sp.)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Nazamid Saari, Ph.D.
Associate Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairman)

Foo Hooi Ling, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

Gulam Rusul Rahmat Ali, Ph.D.
Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

Son Radu, Ph.D.
Associate Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

SHAMSHER MOHAMAD RAMADILI, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 JAN 2003

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Foo Hooi Ling, Ph.D.
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Chairperson)

Gulam Rusul Rahmat Ali, Ph.D.
Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

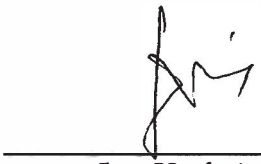
Son Radu, Ph.D.
Associate Professor,
Faculty of Food Science and Biotechnology
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 13 MAR 2003

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



Lee Yock Ann

Date: 20/1/03

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

A	Absorbency
A	Adenine
Aba	Aminibutyricacid
Au	Activity unit
Bu	Bacteriocin unit
BSA	Bovine Serum Albumin
C	Cytosine
°C	Degree Celsius
CD-ELISA	Competitive direct -ELISA
CFNS	Cell Free Neutralised Supernatant
CFS	Cell Free Supernatant
CI-ELISA	Competitive Indirect-ELISA
CM-cellulose	CarboxylMethyl-cellulose
Dha	didehydroalanine
β-meDha	β-methyldidehydroalanine
DNA	Deoxyribonucleic acid
E.	*Enterococcus*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAD	Flavoprotein
FDA	Food and Drug Administration
FPLC	Fast Protein Liquid Chromatography
g	G-force
G	Guanine
GTE	Glucose-Tris-EDTA
HIC	Hydrophobic Interaction Chromatography
IEF	Isoelectric Focusing
kDa	Kilo dalton
kbp	Kilobase-pair
LAB	Lactic Acid Bacteria
LB	Lubria Bertani
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
ml	Mililiter
µl	Microliter
mM	Milimolar
µM	Micromolar
Mr	Molecular Mass
M	Molar
min	Minute
MRS	De Man, Rogasa, Sharpe
NaCl	Sodium Chloride
NCI-ELISA	Non Competitive Indirect-ELISA
OD	Optical Density
P.	*Pediococcus*

pI	Isoelectric point
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PVDF	Polyvinyl difluoride
RAPD	Random Amplified Polymorphic DNA
SDS	Sodium Dodecyl Sulphate
subsp.	*Subspecies*
T	Thymine
TBE	Tris-Boric-EDTA
TCA	Trichloroacetic Acid
UV	Ultraviolet
V	Volt
WHO	World Health Organisation
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Lactic Acid Bacteria (LAB) appear to be a group of unique bacteria, which are granted "Generally Recognised As Safe (GRAS)" status and have been used traditionally as food-grade bacteria food fermentation. Research on LAB has advanced greatly since the last decade due to its important roles in many diverse areas, including biotechnology, nutrition, health and food safety. LAB have been used as starter cultures in the production of various fermented foods and beverages, for instance cheese, yoghurt, fermented sausage, silage, sourdough, beer and wine etc. The potential and ability of LAB to produce several interesting metabolites such as organic acids, enzymes, antimicrobial substances, exopolysaccharides and probiotic properties have attracted the attention of many researcher.

Bacteriocins are natural proteinaceous antimicrobial compounds produced by a large and diverse group of LAB. It possesses antibacterial activity towards other but closely related bacteria. Their proteinaceous nature implies that the bacteriocin are possible degraded in gastrointestinal tracts of man and animals and thus proved to be an excellent candidate as biopreservatives to improve the safety of various fermented or non-fermented foods.

Four distinct classes of bacteriocins have been categorised. The class I bacteriocins are Lantibiotic which are small peptides that have been differentiated from other bacteriocins by their content of didehydroamino acid and thioether amino